The NAD-Booster Nicotinamide Riboside Potently Stimulates Hematopoiesis through Increased Mitochondrial Clearance

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Title: The NAD-booster nicotinamide riboside potently stimulates hematopoiesis through increased mitochondrial clearance

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Supplementary Materials:

- **Supplementary Figure S1:** NA/NAM-supplementation does not cause hematopoietic progenitor expansion.
- **Supplementary Figure S2:** NA/NAM-supplementation does not cause hematopoietic progenitor expansion.
- **Supplementary Figure S3:** NR exerts its effect through the NR/Nrk/NMN axis.
- **Supplementary Figure S4:** NR in vitro affects mitochondrial mass and ΔΨm in HSCs.
- **Supplementary Figure S5:** NR improves blood reconstitution and stem cell function of human CD34+ cells.
- **Supplementary Figure S6:** NR induces autophagy, mitophagy and mitochondrial stress.
- **Supplementary Figure S7.** NR induces mitochondrial stress in K562 cells in a dose dependent manner and does not exhaust HSC function in secondary transplantation.

Supplementary Materials:

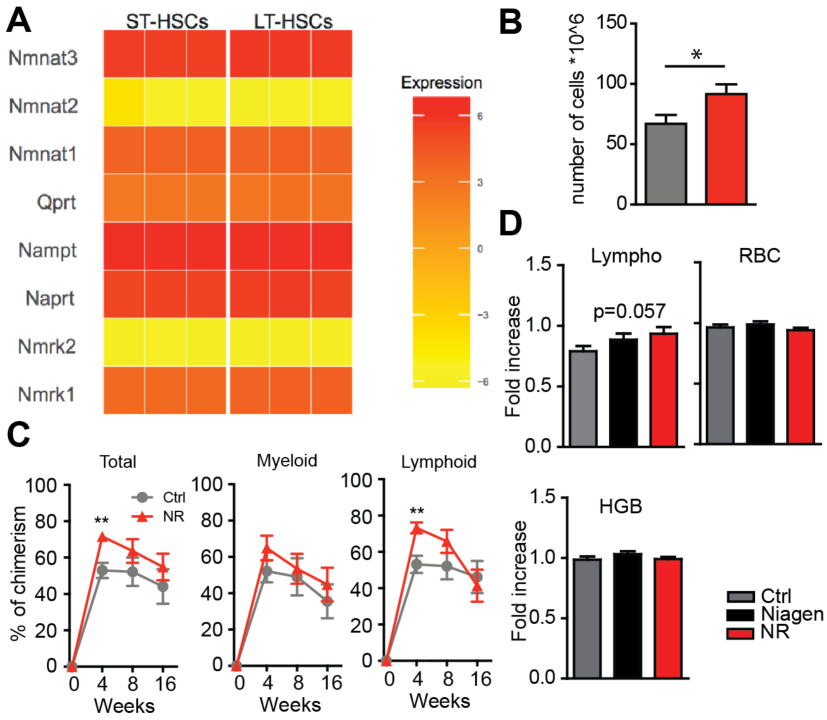


Figure S1. Transcriptome analysis of the salvage pathway genes and influence of NR on hematopoietic system. A, Key genes of the NAD salvage pathway are both expressed in LT-HSCs and ST-HSCs freshly sorted from the bone marrow of 10 week-old C57BL6 females. *n=3.* **B,** BM cellularity of mice fed for 1 week with ctrl or NR diet (legs and hips)*, n=5*. **C ,** Blood chimerism of mice transplanted with BM derived from ctrl or NR fed mice was assessed at 4, 8 and 16 weeks, *n=10.* Readings at 16 weeks show no significant difference, indicating that the LT-HSC pool is neither expanded nor exhausted after NR treatment**,** *n=10.* **D,** Analysis of peripheral blood for Lymphocytes, Red blood cells (RBC) and hemoglobin (HGB) in mice fed with ctrl, Niagen or NR diet, *n=5. Student's t test *P<0.05, **P<0.01, ***P<0.001.*

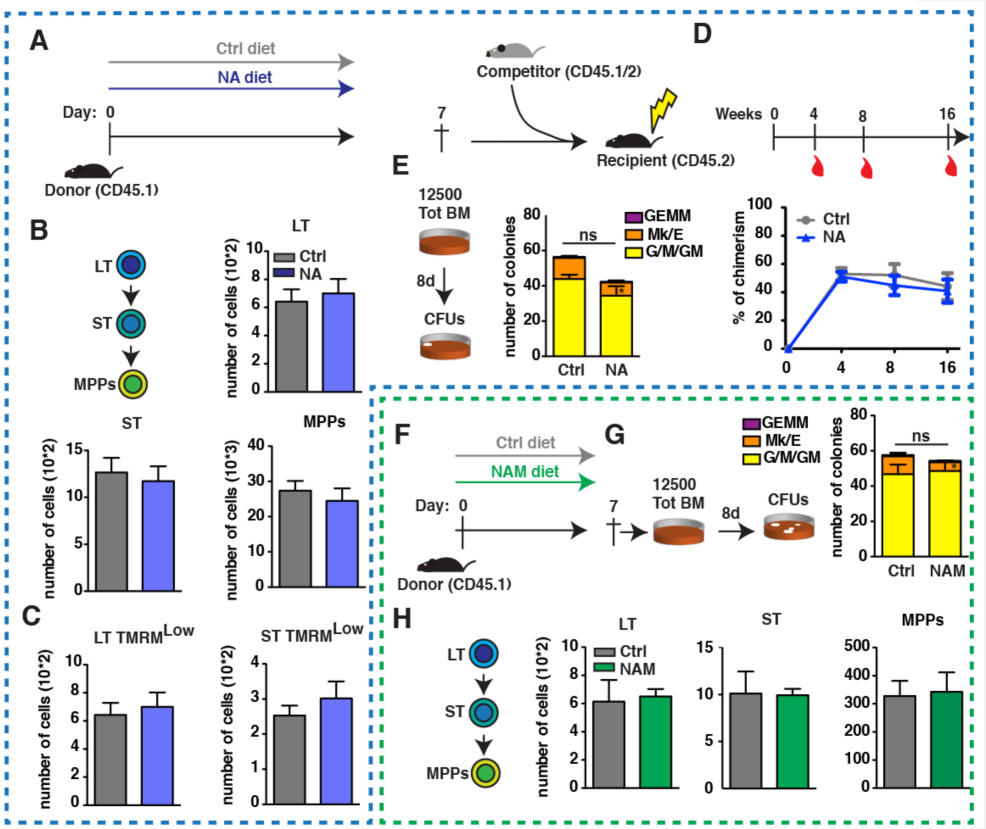


Figure S2. NA/NAM-supplementation does not cause hematopoietic progenitor expansion. A, Mice were fed 1 week with control or NA diet, mice were sacrificed for BM analysis and primary transplant. **B,** Phenotypic analysis of the different BM hematopoietic stem/progenitor compartments from ctrl or NA fed mice by flow cytometry, *n=5.* **C ,** LT and ST-HSC mitochondrial potential measured by TMRM staining from BM of ctrl or NR diet fed mice, *n=5.* **D,** Blood chimerism of mice transplanted with BM derived from ctrl or NA fed mice was assessed at 4, 8 and 16 weeks, *n=10.* **E,** 12,500 BM cells from ctrl- or NA-fed mice were plated in Methocult for colony forming CFUs analysis 8 days after plating, *n=5.* **F,** Mice were fed 1 week with control or NAM diet, mice were sacrificed for BM analysis and secondary transplants. **G,** 12,500 BM cells from ctrl- or NAM-fed mice were plated

in Methocult for colony forming CFUs analysis 8 days after plating, *n=5.* **H,** Phenotypic analysis of the different BM hematopoietic stem/progenitor compartments from ctrl or NAM fed mice by flow cytometry, *n=5. .. Student's t test *P<0.05, **P<0.01, ***P<0.001.*

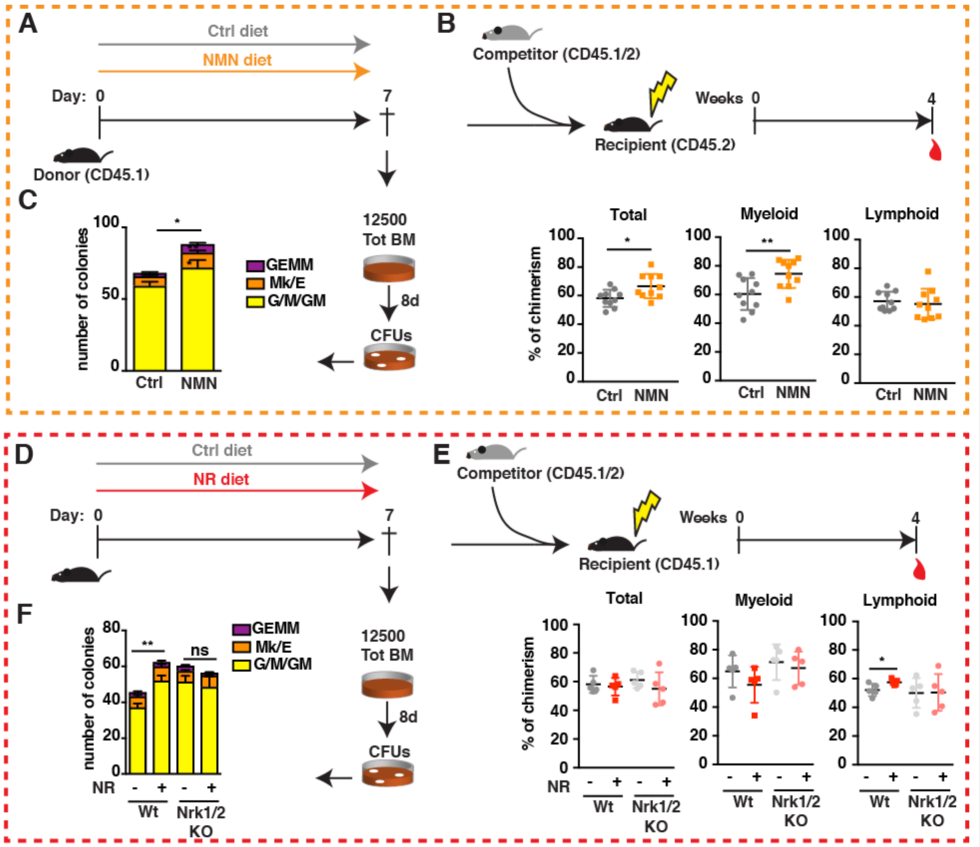


Figure S3. NR exerts its effect through the NR/Nrk/NMN axis. **A,** Mice were fed 1 week with control or NMN diet, mice were sacrificed for BM analysis and secondary transplants. **B,** Blood chimerism of mice transplanted with BM derived from ctrl or NMN fed mice was assessed at 4 weeks, *n=10.* **C,** 12,500 BM cells from ctrl- or NMN-fed mice were plated in Methocult for colony forming CFUs analysis 8 days after plating, *n=5.* **. D,** Nrk1-/-;Nrk2-/- double KO mice were fed 1 week with control or NR diet, mice were sacrificed for BM analysis and secondary transplants. **E,** Blood chimerism of mice transplanted with BM derived from wt or Nrk1/2 KO mice fed with ctrl or NR diet was assessed at 4 weeks, *n=10.* **F,** 12,500 BM cells from wt or Nrk1/2 KO mice fed with ctrl or NR diet were plated in Methocult for colony forming CFUs analysis 8 days after plating, *n=5. Student's t test *P<0.05, **P<0.01, ***P<0.001.*

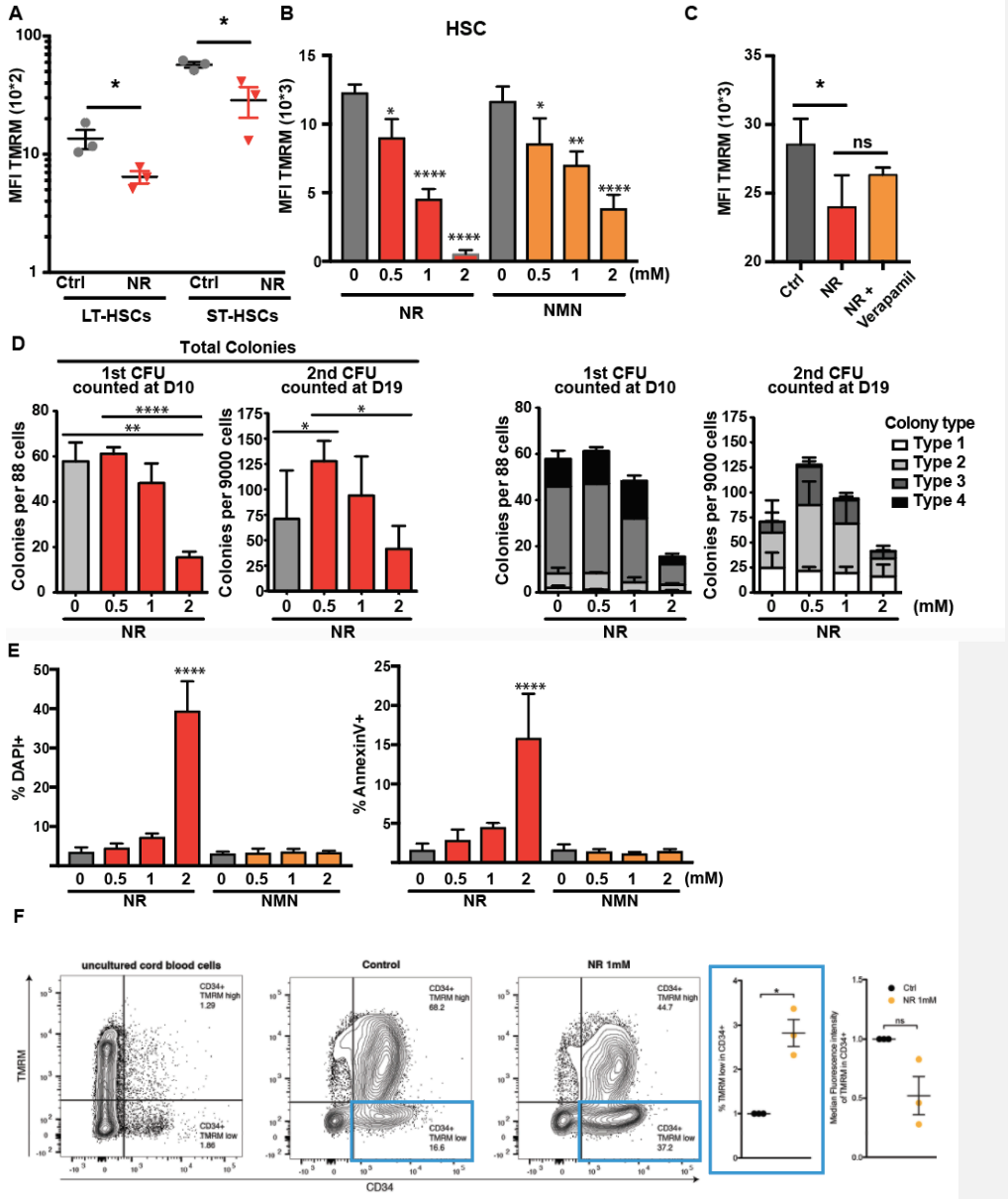
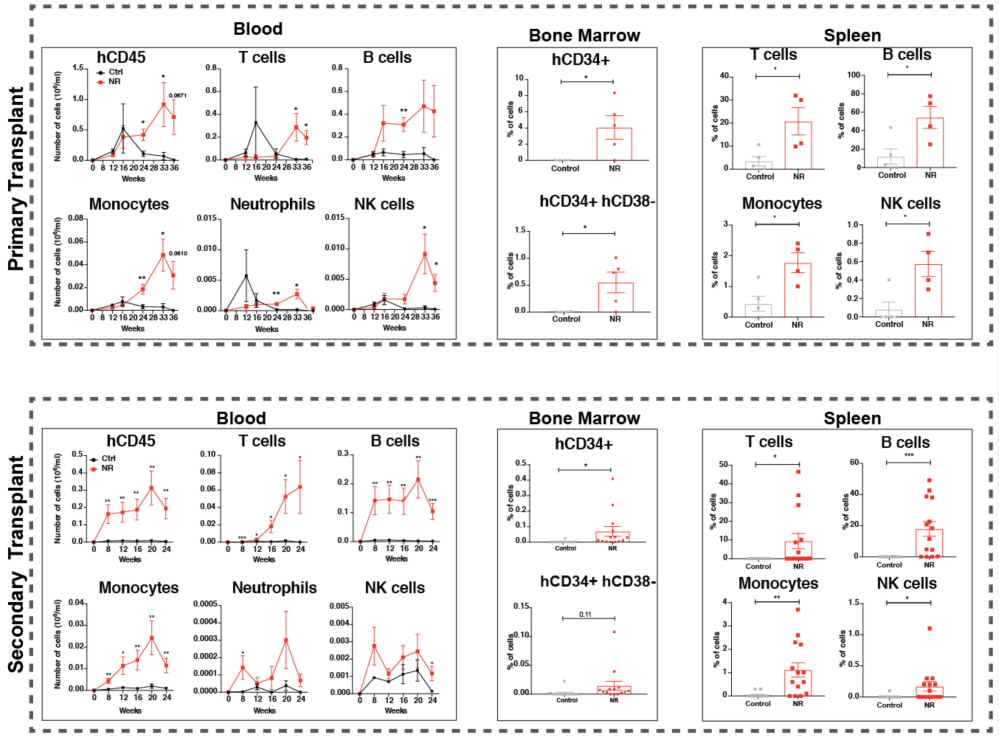


Figure S4. NR *in vitro* affects mitochondrial mass and ▯▯m in HSCs. A, LT- and ST-HSCs were FACS sorted and treated in vitro for 48 hours with 0.5 mM NR (A,) or increasing concentrations of NR or NMN (B,), then stained with

TMRM and analyzed by FACS. **C,** HSCs were cultured for 48h with NR. The calcium dependent ABC pump inhibitor verapamil was added at 50μM 1 h before TMRM staining and subsequent flow cytometric analysis. There was no significant difference on the NR-mediated decrease in TMRM signal in presence or absence of verapamil. **D,** HSCs cultured with NR for 48h, were plated in M3434 methylcellulose and their long-term colony-forming capacity was analyzed in secondary CFU assays. Note that there is no differences in the primary CFU and that the highest NR concentration of 2mM was toxic to murine HSCs, but not to human CD34+ progenitors (Figure 3A). **E,** HSCs cultured with NR or NMN at different concentrations, stained with DAPI and Annexin V, and analyzed by FACS. **F,** Cord blood derived human CD34+ cells were cultured for 2 days in the presence or absence of NR as in Fig. 3A. Shown is the experimental replicate for cord blood in a different laboratory with completely independent donors (upper panel & lower panel). Both the fold change in the $TMRM^{low}$ population (labeled in blue) and the TMRM MFI of total CD34+ were quantified. *n=3.Student's t test *P<0.05, **P<0.01, ***P<0.001*



Supplementary Figure S5: NR improves blood reconstitution and stem cell function of human CD34+ cells. Fetal derived human CD34+ cells were cultured for 5 days in the presence or absence of NR and transplanted in irradiated NSG mice. Reconstitution of different blood lineages (Blood panels) was followed over time in primary (1^0 Transplant) and secondary (2^0 Transplant) recipient mice. At the endpoint hematopoietic progenitors (CD34+ and CD34+CD38-) and blood lineages were quantified in the bone marrow and spleen of recipients' mice respectively. *n=6 & n=12 . Student's t test *P<0.05, **P<0.01, ***P<0.001.*

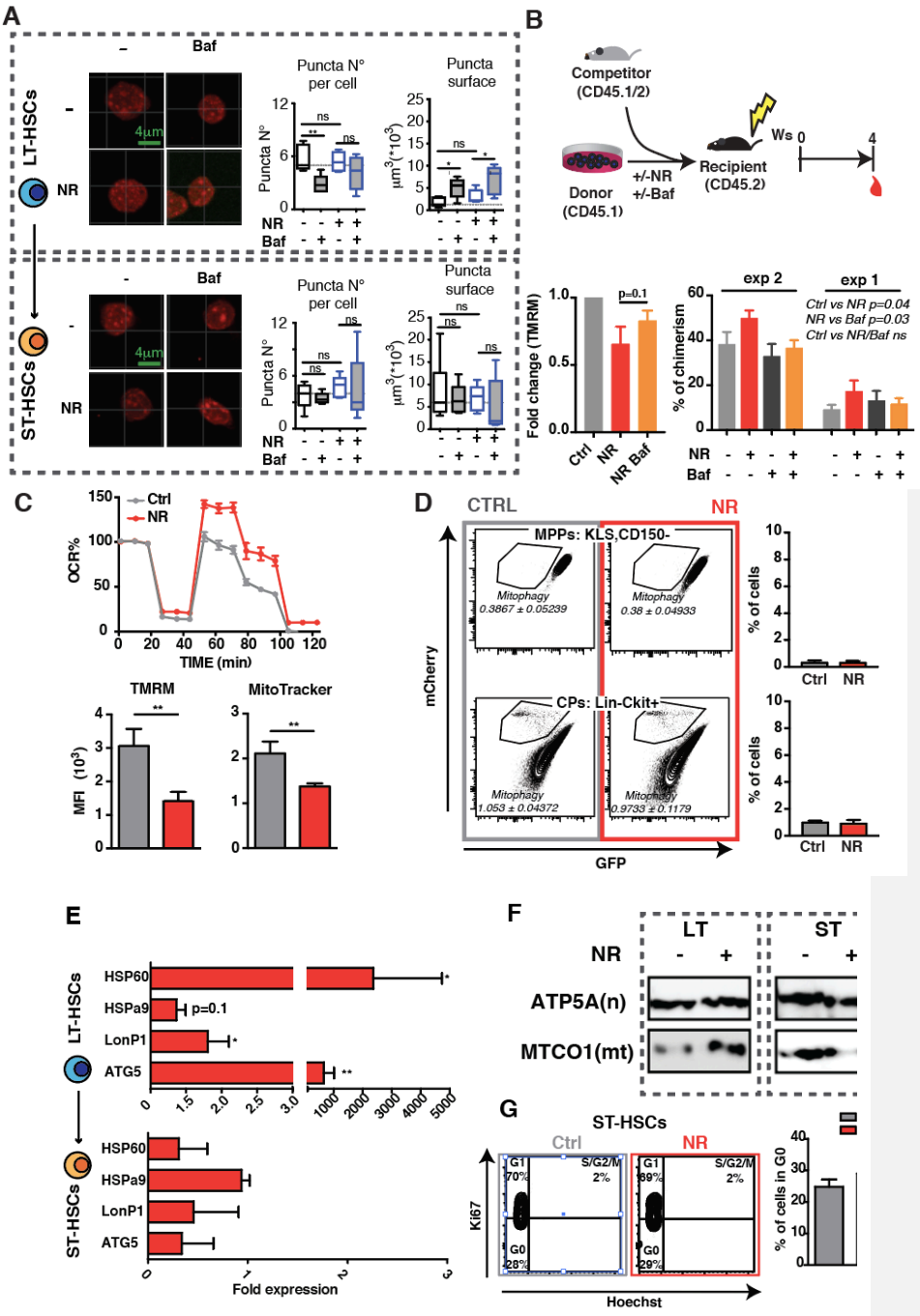
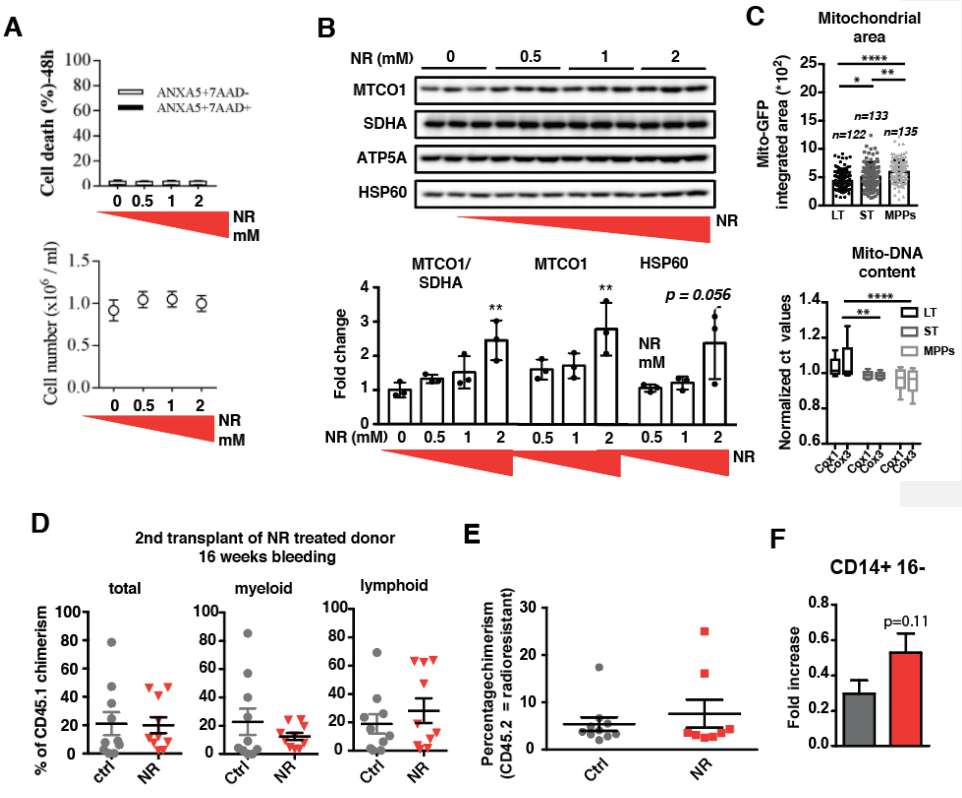

A
Baf
LT-HSCs
NR
4µm
Puncta N° per cell
Puncta surface
Puncta N°
µm3(*10³)
ns
NR
Baf
ST-HSCs
B
Competitor (CD45.1/2)
Donor (CD45.1)
+/-NR +/-Baf
Recipient (CD45.2)
Ws
0
4
Fold change (TMRM)
p=0.1
Ctrl
NR
NR Baf
% of chimerism
exp 2
exp 1
Ctrl vs NR p=0.04
NR vs Baf p=0.03
Ctrl vs NR/Baf ns
C
OCR%
TIME (min)
Ctrl
NR
TMRM
MitoTracker
MFI (10³)
D
CTRL
NR
MPPs: KLS,CD150-
CPs: Lin-Ckit+
Mitophagy
0.3867 ± 0.05239
0.38 ± 0.04933
1.053 ± 0.04372
0.9733 ± 0.1179
mCherry
GFP
% of cells
E
LT-HSCs
ST-HSCs
HSP60
HSPa9
LonP1
ATG5
p=0.1
Fold expression
F
LT
ST
NR
ATP5A(n)
MTCO1(mt)
G
ST-HSCs
Ctrl
NR
G1
S/G2/M
G0
Ki67
Hoechst
% of cells in G0

Figure S6. NR induces autophagy, mitophagy and mitochondrial stress. A, Confocal analysis of HSCs exposed to NR, autophagy blocker Bafilomycin, or both. Cells were stained with Lc3B antibody and number and size of autophagosomes was measured. (Upper panels LT-HSCs; Lower panel ST-HSCs). **B,** LT-HSCs (CD45.1) were cultured for 2 days in the presence of absence of NR and Bafilomycin (Baf). ΔΨm of HSCs was then analyzed by flow cytometry with TMRM staining in presence or absence of bafilomycin (lower left, n=4). LT-HSCs were cultured for a total of two days and transplanted into lethally irradiated mice (CD45.2) together with total bone marrow competitor cells (CD45.1/2). Engraftment capability was measured by blood analysis in 2 independent experiments (lower right). Statistics are for the combined experiments, which are shown separately because the baseline engraftment was very different for both cohorts. **C,** Splenocyte-derived cells were cultured with NR over 3 days. Respiratory capacity was measured by seahorse (upper), ΔΨm by TMRM staining (lower left) and mitochondrial mass by mitotracker staining (lower right) **D,** Mito-QC mice were fed for 1 week with control or NR diet. Percentage of cells undergoing mitophagy was quantified. Downstream hematopoietic progenitors, MPPs (KLS CD150-) and CPs (Lin- cKit+) showed lower mitophagy rates as compared to the most primitive hematopoietic compartments and most importantly, their mitophagy rates were not affected by NR supplementation. **E,** Backgating analysis of Mito-QC cells. In the Mitophagy analysis of LT and ST-HSCs the $mCherry^{high}$ population (left panels) was excluded, as it represented PI^{interm} cells which backgated away from the core HSC population both in the FSC and KLS gates. **F,** Gene expression analysis of ST or LT-HSCs isolated from the BM of NR or control diet fed C57BL6 mice, *n=4*. **G,** Mito-nuclear protein imbalance in HSCs FACS sorted from mice fed control vs. NR diet for 7 day. Western blotting protein analysis of mitochondrial proteins encoded by nuclear subunit (ATP5a) and mitochondrial subunit (MTCO1) of the oxidative phosphorylation complex (see text for interpretation, data derived from the pooled bone marrow of 32 mice). **G,** Cell cycle analysis of ST-HSCs derived from NR or control diet fed mice, *n=5. Student's t test *P<0.05, **P<0.01, ***P<0.001.*



Supplementary Figure S7: NR induces mitochondrial stress in K562 cells in a dose dependent manner and does not exhaust HSC function in secondary transplantation.

A, Cell death was assessed by flow cytometry using annexin V (ANXA5) and 7AA D double staining after 48 hours of NR exposure. Absolute cell number was determined by flow cytometry using absolute counting beads. **B,** western blot quantification of NR-driven mito-nuclear protein unbalance (MTCO1/SDHA) and NR-driven induction of UPRmt chaperone HSP60. **C,** Mito-EGFP derived hematopoietic stem and progenitor cells were analyzed for their mitochondrial content. Confocal analysis of single cell integrated EGFP signal was used to quantify mitochondrial area (upper panel). Mitochondrial DNA content was analyzed by QPCR targeting 2 different mitochondrial genes (Cox1 and Cox3). Values are expressed as normalized ct that inversely correlated to the DNA

content (lower panel). **D,** Analysis of radioresistant BM (recipient, CD 45.2+) of transplanted mice with BM derived from ctrl or NR diet fed mice, *n=10.*. **E,** Secondary transplants were performed with total bone marrow from mice having received limiting-dose transplantation while treated with NR or control diet (Figure 7C-D), then analyzed at 16 weeks for persistence of donor CD45.1 stem cell function. **F,** Analysis of CD14+ CD16- monocytes of peripheral blood of humanized mice fed for 7 days with ctrl or NR diet (gated on hCD45), *n=5. Student's t test *P<0.05, **P<0.01, ***P<0.001 and Two-way ANOVA *P<0.05, **P<0.01, ***P<0.001.*